Rest of the LabBook

14 September 2016

The promoter region of the vectors pSEVA 224 and pSEVA 2311 were amplified by PCR with Q5 DNA polymerase with primers allowing to add the prefix and suffix regions at both sides of the amplified region.

Mix:

- 10 µL Q5 reaction buffer 5X
- 1 µL dNTP mix
- 2.5 µL 10 µM Forward Primer (iG063)
- 2.5 µL 10 µM Reverse Primer (iG064)
- 1 µL DNA template
- 0.5 µL Q5 DNA polymerase
- 32.5 µL Nuclease-free water (qs 50 µL)

PCR program:

1) 98°C 30s
2) 95°C 30s
3) 55°C 30s
4) 72°C 1 min
   35 repeats of the steps bloc from 2) to 4)
5) 72°C 2 min
6) 10°C infinite

➔ After checking on agarose 1% gel, we determined that the fragments were successfully amplified.

Digestion

The amplified fragments were digested by EcoRI and PstI during 1h at 37°C. The reaction was performed in NEB CutSmart buffer.

The digested mixes were purified by using the kit Wizard SV Gel and PCR Clean-Up System (Ref: A9282, Promega).

15 September 2016

Ligation

The 2 fragments were then ligated separately in linear pSB1C3 provided by IGEM Headquarters in a ratio 5:1.

The ligation mix was used for transformation in *E. coli* DH5-alpha.

Transformation

- Thaw 25 µL chemo-competent DH5-alpha *E. coli*
- Add 3 µL DNA (mix ligation)
- Incubation 20 min on ice
- Heat shock: 45s at 42°C
- Incubation 3 min on ice
- Add 225 µL of LB medium
- Incubation 1h at 37°C with shaking
- Spread 120 µL on plates LBC
- Incubation overnight 37°C

16 September 2016

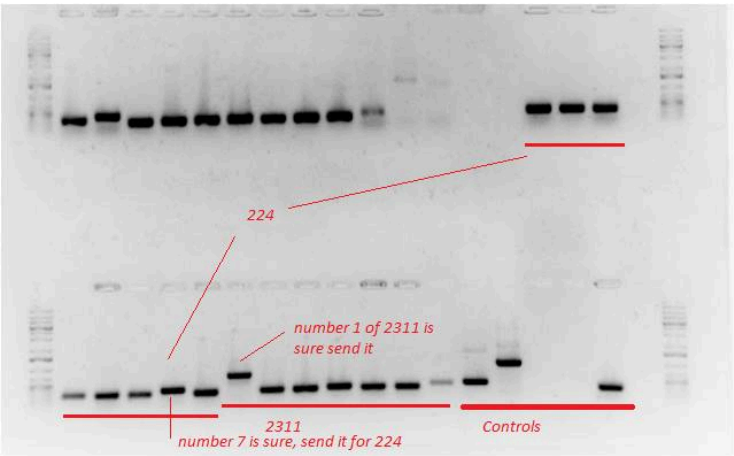
Colonies PCR

Mix (25 µL total volume reaction):

- 12.5 µL DreamTaq PCR Mastermix 2X (ThermoScientific)
- 2.5 µL 10X DreamTaq Green Buffer (ThermoScientific)
- 0.25 µL Forward Primer (0.5 µM) iG001
- 0.25 µL Reverse Primer (0.5 µM) iG002
- 9.5 µL H2O filtrated (qs 25 µL)

+ clones

Agarose gel 1%



Precultures

Positive clones selected from the agarose gel results were picked and used to inoculate 3 mL of LBC (25 µg/mL chloramphenicol) medium.

20 September 2016

Precultures

3 mL LB medium in 15 mL Falcon tube+ 1µL P. putida KT2440 from -80°C stock

Incubation 30°C overnight with shaking

21-23 September 2016

Growth experiments

Stock in 50% glycerol stored at -80°C

Experiments performed in triplicates on 96 wells plate with LB medium as blank.

The plate was incubated at 30°C with shaking during 20h after inoculating with 1/1000 dilution of preculture.

The OD at 600 nm was read every 20s.

The experiments were repeated 3 consecutive days from fresh precultures started the day before.

The results were sent to Imperial College IGEM team for collaboration along with the results of carbon source tests performed in July.

4 October 2016

Gibson assembly 2nd step

pSB1C3 IGEM Headquarters 110.3 ng/µL

pSB1C3 mRFP 14.1 ng/µL

Mix Gibson from the 1st step giving the operon:

1) PCT2+PhaC3 65.5 ng/µL
2) PCT2+PhaC4 60.8 ng/µL
3) PCT2+PhaC3 63.4 ng/µL
4) PCT2+PhaC4 65.7 ng/µL

Gibson ratio 1:1

Vector amplified from pSB1C3-mFRP	1)	Vector	3.40 µL (50 ng)
		Insert (mix 1)	1.21 µL
		H2O (qs 10 µL)	5.40 µL
	2)	Vector	3.40 µL (50 ng)
		Insert (mix 2)	1.30 µL
		H2O (qs 10 µL)	5.30 µL
	3)	Vector	3.40 µL (50 ng)

		Insert (mix 3)	1.25 µL
		H2O (qs 10 µL)	5.35 µL
	4)	Vector	3.40 µL (50 ng)
		Insert (mix 4)	1.20 µL
		H2O (qs 10 µL)	5.40 µL
Linear vector pSB1C3 provided by IGEM Headquarters	5)	Vector	0.91 µL (100 ng)
		Insert (mix 1)	2.42 µL
		H2O (qs 10 µL)	6.68 µL
	6)	Vector	0.91 µL (100 ng)
		Insert (mix 2)	2.60 µL
		H2O (qs 10 µL)	6.49 µL
	7)	Vector	0.91 µL (100 ng)
		Insert (mix 3)	2.50 µL
		H2O (qs 10 µL)	6.60 µL
	8)	Vector	0.91 µL (100 ng)
		Insert (mix 4)	2.41 µL
		H2O (qs 10 µL)	6.68 µL

Add 10 µL NEB HiFi DNA Assembly Mastermix 2X
Incubation 50°C, 15 min
Cool on ice

Transformation

- Thaw 50 µL chemo-competent DH5-alpha *E. coli*
- Add 2 µL DNA (mix Gibson)
- Incubation 30 min on ice
- Heat shock: 30s at 42°C
- Incubation 2 min on ice
- Add 950 µL NEB medium (#B9020S)
- Incubation 1h at 37°C with shaking
- Centrifugation 7000 rpm 1 min
- Resuspend cells with rest of supernatant after throwing away the most of it
- Spread all the content on plates LBC
- Incubation overnight 37°C

7 October 2016

Solubilisation of IDT gBlocks in 100 µL of nuclease-free water and short centrifugation

Incubation 50°C during 20 min

Vortexing and then, short centrifugation again

PCR of gBlock genes

Mix:

- 10 µL Q5 reaction buffer 5X
- 1 µL dNTP mix
- 2.5 µL 10 µM Forward Primer (iG063)
- 2.5 µL 10 µM Reverse Primer (iG064)
- 1 µL DNA template
- 0.5 µL Q5 DNA polymerase
- 32.5 µL Nuclease-free water (qs 50 µL)

Mix for 5 reactions

PCR program:

7) 98°C 30s
8) 95°C 30s
9) 55°C 30s
10) 72°C 1 min
    35 repeats of the steps bloc from 2) to 4)
11) 72°C 2 min
12) 10°C infinite

Colony PCR on Gibson transformation

- 12.5 µL DreamTaq Green PCR Master Mix 2X (Thermo Scientific, #K1081)
- 1.25 µL 10 µM Forward Primer (iG001)
- 1.25 µL 10 µM Reverse Primer (iG002)
- 10 µL Nuclease-free water (qs 25 µL)

+ clone

Mix for 23 reactions

PCR program:

1) 95°C 5 min
2) 95°C 30s
3) 50°C 30s
4) 72°C 3 min
    30 repeats of the steps bloc from 2) to 4)
5) 72°C 10 min
6) 10°C infinite

8 October 2016

➔ Problem with several PCR reaction tubes → we do them again

Colony PCR on Gibson transformation

- 12.5 µL DreamTaq Green PCR Master Mix 2X (Thermo Scientific, #K1081)
- 1.25 µL 10 µM Forward Primer (iG001)

- 1.25 µL 10 µM Reverse Primer (iG002)
- 10 µL Nuclease-free water (qs 25 µL)

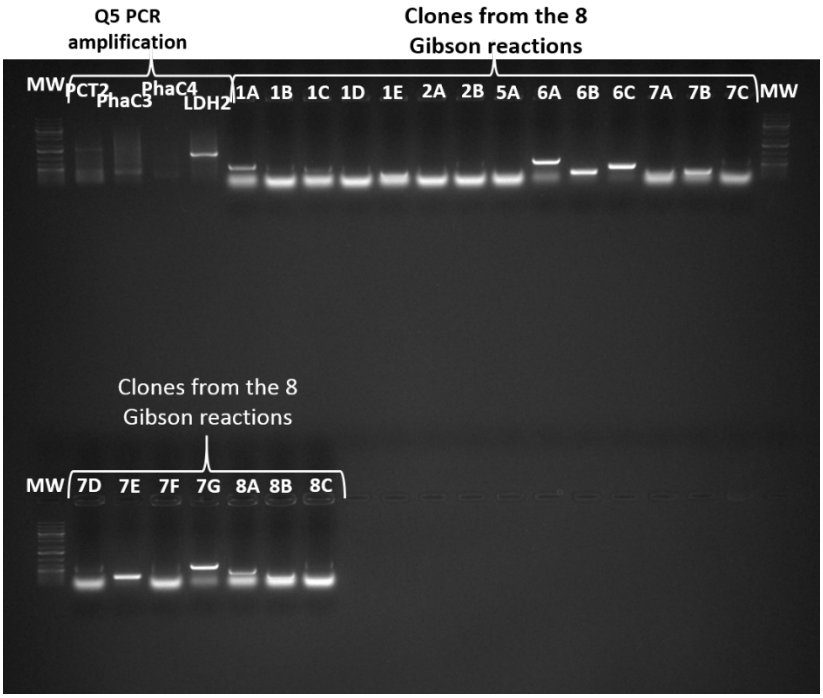
+ clone

Mix for 9 reactions

PCR program:

1) 95°C 5 min
2) 95°C 30s
3) 50°C 30s
4) 72°C 3 min
   30 repeats of the steps bloc from 2) to 4)
5) 72°C 10 min
6) 10°C infinite

Agarose gel 1%



9 October 2016

Pouring of 46 LBC (10 µg/mL chloramphenicol) plates + 5 LBK (50 µg/mL kanamycin) plates + 2 LBS (50 µg/mL spectinomycin) platesx

10 October 2016

Concentration measurements:

LDH 7.10: 37.7 ng/µL
PCT 7.10: 29.8 ng/µL

PhaC3 7.10: 35.7 ng/µL
PhaC4 7.10: 40.3 ng/µL

LDH 3.09: 16.3 ng/µL
PCT 3.09: 7.1 ng/µL
PhaC3 3.09: 8.2 ng/µL
PhaC4 3.09: 9.0 ng/µL

LDH 21.09: 7.9 ng/µL
PCT 21.09: 7.8 ng/µL
PhaC3 21.09: 7.0 ng/µL
PhaC4 21.09: 7.3 ng/µL

LDH 30.08: 8.8 ng/µL
PCT 30.08: 7.7 ng/µL
PhaC3 30.08: 11.0 ng/µL
PhaC4 30.08: 8.1 ng/µL

15 October 2016

Digestion PhaC3_only and PCT2_only

- 30 µL IDT gBlaock gene
- 5 µL buffer NEB CutSmart 10X
- 1 µL EcoRI
- 1 µL SpeI
- 13 µL nuclease-free water (qs 50 µL)

Incubation 1h 37°C

DNA clean & concentrator-5 kit (Zymo Research, D4003S)
DNA binding buffer added with 5:1 ratio
1 min incubation after adding 20 µL of nuclease-free water before centrifugation for elution

Ligation

	Quick ligase	T4 DNA ligase
pSB1C3 digested by E+S (from clone B → 13.3 ng/µL)	6 µL	6 µL
gene digested by E+S	3 µL	3 µL

Buffer	10 µL Quick ligase buffer 2X (NEB)	2 µL T4 DNA ligase buffer 10X (NEB)
ligase	1 µL Quick ligase (NEB)	1 µL T4 DNA ligase
H2O (nuclease-free water)	-	8 µL

Transformation ligation mixes in pSB1C3

- Thaw 50 µL chemo-competent DH5-alpha *E. coli*
- Add 2 µL DNA (mix ligation)
- Incubation 20 min on ice
- Heat shock: 45s at 42°C
- Incubation 3 min on ice
- Add 450 µL LB medium
- Incubation 1h at 37°C with shaking
- Centrifugation 7000 rpm 3 min
- Throw away supernatant
- Resuspend cells with rest of supernatant (around 50 µL)
- Spread all the content on plates LBC (10 µg/mL chloramphenicol)
- Incubation overnight 37°C

Transformation ligation mixes in pSEVA 224, 424 or 2311

- Thaw 50 µL chemo-competent DH5-alpha *E. coli*
- Add 2 µL DNA (mix ligation)
- Incubation 20 min on ice
- Heat shock: 45s at 42°C
- Incubation 3 min on ice
- Add 450 µL LB medium
- Incubation 1h at 37°C with shaking
- Centrifugation 7000 rpm 3 min
- Throw away supernatant
- Resuspend cells with rest of supernatant (around 50 µL)
- Spread all the content on plates LBK (50 µg/mL kanamycin) for pSEVA 224 or 2311 or LBS (50 µg/mL spectinomycin) for pSEVA 424
- Incubation overnight 37°C

DNA PCR clean-up kit (NEB) for gBlock genes amplified by PCR

16 October 2016

➔ Our strain of DH5-alpha bacteria seems already resistant to spectinomycin → test by preculture in 3 mL LBS (50 µg/mL spectinomycin) liquid medium

Concentration of amplified gBlocks after PCR clean-up

PhaC4: 86.6 ng/µL
PhaC3: 120.6 ng/µL
LDH2: 202.5 ng/µL
PCT2: 154.6 ng/µL

Digestion by E+P of amplified genes

	PhaC3	PhaC4	PCT2	LDH2
DNA genes	7 µL	7 µL	5 µL	4 µL
Buffer NEB CutSmart	5 µL	5 µL	5 µL	5 µL
EcoRI	1 µL	1 µL	1 µL	1 µL
PstI	1 µL	1 µL	1 µL	1 µL
H2O nuclease-free	36 µL	36 µL	38 µL	39 µL

DNA clean & concentrator-5 kit (#D4003S Zymo Research)
Ratio 5:1 of DNA binding buffer
Incubation 1 min with 20 µL of nuclease-free water before centrifugation for elution

Precultures

2 mL LBC (25 µg/mL chloramphenicol) + clones from transformation of DH5-alpha E. coli with ligation mixes of pSB1C3 with PhaC3_only or PCT2_only

17 October 2016

Miniprep

GenElute Plasmid Miniprep Kit (Sigma, PLN350-1KT)

3 clones for PhaC3 and 14 clones for PCT2

18 October 2016

Colony PCR on the last trials of insertion of PhaC3, PhaC4, PCT2, LDH2, PhaC3_only and PCT2_only inside pSB1C3 and of insertion of PhaC4, PCT2 and LDH2 inside pSEVA224 and pSEVA2311 in process.

19 October 2016

Characterization of pSEVA2311 in process.